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Role of Ran GTPase in regulating milk protein synthesis signaling pathways in bovine mammary epithelial cells

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ABSTRACT

RAN, A member of the small GTPase superfamily, is involved in various cellular signaling pathways. That regulates cell cycle progression and protein synthesis. This study aimed to investigate how Ran influences key signaling pathways associated with cell survival, proliferation, and milk protein synthesis (mTOR and Jak2/Stat5). Tissue samples were obtained from six mid-lactation Holstein dairy cows with high genetic merit. Western blot analysis was performed to determine the expression levels of mTOR, phosphorylated mTOR (p-mTOR) , Stat5 and phosphorylated Stat5 (p-Stat5) after Ran overexpression after Ran timely and stable transfection, the β-casein secreted by bovine mammary epithelial cells (BMECs) was quantified using HPLC and Cell viability was assessed with CASY cell counter. The results showed that after Ran Overexpression significantly enhanced cell viability and increased the expression of mTOR , p-mTOR , Stat5 and p-Stat5. The cell viability was significantly increased, furthermore the expression of mTOR, p-mTOR, Stat5, and p-Stat5 were also significantly increased. Thus, these findings suggest that Ran positively regulates the Jak2/Stat5 pathway and mTOR Signaling pathways, thereby promoting milk protein synthesis in BMECs.

دور Ran GTPase في تنظيم مسارات إشارات تخليق بروتين الحليب في الخلايا الظهارية الثديية البقرية

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الخلاصة

RAN، أحد أعضاء عائلة GTPase الصغيرة، يشارك في مسارات إشارات خلوية مختلفة. ينظم تقدم دورة الخلية وتخليق البروتين. هدفت هذه الدراسة إلى التحقيق في كيفية تأثير Ran على مسارات الإشارات الرئيسية المرتبطة ببقاء الخلية والتكاثر وتخليق بروتين الحليب (mTOR و Jak2 / Stat5). تم الحصول على عينات الأنسجة من ستة أبقار هولشتاين حلوب في منتصف فترة الرضاعة ذات قيمة وراثية عالية. تم إجراء تحليل لطخة ويسترن لتحديد مستويات التعبير عن mTOR و mTOR المفسفر (p-mTOR) و Stat5 و Stat5 المفسفر (p-Stat5) بعد الإفراط في التعبير عن Ran ,بعد انتقال Ran في الوقت المناسب والمستقر، تم تحديد كمية β-casein التي تفرزها الخلايا الظهارية الثديية البقرية (BMECs) باستخدام HPLC وتم تقييم قابلية بقاء الخلايا الحية باستخدام عداد خلايا CASY. أظهرت النتائج أنه بعد الإفراط في التعبير عن Ran، عزز بشكل كبير قابلية الخلية للحياة وزاد من التعبير عن mTOR و p-mTOR و Stat5 و p-Stat5. ازدادت قابلية الخلايا للبقاء بشكل ملحوظ، كما ازداد التعبير عن mTOR وp-mTOR وStat5 وp-Stat5 بشكل ملحوظ. وبالتالي، تشير هذه النتائج إلى أن Ran ينظم بشكل إيجابي مسار Jak2/Stat5 ومسارات إشارات mTOR، مما يعزز تخليق بروتين الحليب في خلايا BMEC.

الكلمات المفتاحية: β –casein , signaling pathways, BMECs, Stat5, Ran, mTOR .

INTRODUCTION

Numerous factors influence the production of milk proteins in Bovine mammary epithelial cells (BMECs); the synthesis of β-casein (CSN2) is a complex process, as is the milk protein synthesis pathway (Bionaz and Loor, 2011). Research has shown that the Jak2/Stat5 and mTOR pathways are the primary signaling routes regulating CSN2 production (Khudhair *et al.*, 2015; Zhao *et al.*, 2017). According to recent research, prolactin, IGF-1, insulin, cytokines, and amino acids all influence the mTOR and Jak2/Stat5 pathways, which govern the synthesis of milk proteins (Burgos and Cant, 2010; Burgos *et al*., 2010). Furthermore, activation of Janus kinase (JAK) promotes cell death, differentiation, migration, and proliferation, which are essential for mammary gland formation and lactation according to these cellular linking this more clearly to the role of BMECs for smoother logical flow (Rawlings *et al.*, 2004).

The survival of every eukaryotic organism examined to date depends on Ran (GTP-binding nuclear protein Ran), a small, evolutionarily conserved member of the Ras GTPase superfamily (Boudhraa *et al.*, 2020). A sort of compromised cell function, which includes DNA replication, nuclear membrane improvement, chromatin shape, and RNA export, have been diagnosed by initial exam of the consequences of disruption of the Ran GTPase device (El-Tanani *et al*., 2023; Scott *et al*., 2024). Because Ran mutations interfere with DNA synthesis and nucleocytoplasmic transport, Ran proteins are also implicated in cell cycle progression (Mumtaz *et al*., 2022).

Recent research has proven that Ran is engaged in a number of activities, such as protein synthesis, cell cycle manipulates, and signalling pathways, further to its usual roles in cells (Ning *et al*.,2013; Barrès *et al*., 2019). Stat5 is a major factor controlling the expression of milk protein genes and cell proliferation, which has largely been investigated in mice and bovine (Khatib *et al*., 2008). These reports differ from that of Bionaz and Loor,

2011 who supported a minor role of JAK2/Stat5 signalling for milk protein synthesis in bovine mammary gland *in vivo*. The function of the STAT5 gene and regulatory networks linking Stat5 in the milk production of BMECs remains controversial. The GTPase RAN is an activator of the transcription (JAK/STAT) pathway (He *et al.,* 2021).

Further explore Ran's mechanism of action, this study transiently and stably transfected the Ran gene into cultured primary BMECs and observed its effects on the Jak2/Stat5 and mTOR pathways, as well as on milk protein synthesis and cell viability. These findings provide a deeper understanding of Ran's role in the signaling pathways governing milk protein synthesis.

MATERIALS AND METHODS

Culture and Identification of BMECs

Tissue samples were obtained from six mid-lactation Holstein dairy cows of high genetic merit. Cows were killed after exsanguination and mammary gland tissues were excised aseptically with the agreement of the Animal Care and Use Committee of Northeast Agricultural University. Mammary tissue samples were cut into ~1 cm^3 blocks, excluding connective tissues, frozen in liquid nitrogen and stored at −80°C for later analysis BMECs were cultured using the previously described methods by Tong et al. (2012) and Wang et al. (2014). Cells have been cultivated in Dulbecco's modified Eagle's medium (DMEM:F12) (Gibco, Grand Island, NY) with five milligrammes of insulin (Sigma-Aldrich, Oakville, ON, Canada), a hundred milligrammes of penicillin, a hundred microliters of streptomycin, and 10% fetal bovine serum (FBS, Gibco) introduced. Before further processing, purified cells have been cultivated for 12 hours in serum-unfastened media.

Cytokeratin 18 and β-casein (CSN2) had been detected by way of immunofluorescence to evaluate the purity and ability to synthesize milk protein of these primary BMECs. Briefly, the BMECs on the coverslips were incubated overnight with cytokeratin 18 antibody labelled with FITC (Acris, Hiddenhausen, Germany) and anti-CSN2 primary antibody (Santa Cruz Company) at 1:200 dilutions for 1 h at 37℃and incubated with DAPI for nuclear staining for 15 min at 37℃. The coverslips were visualized by using a confocal laser scanning microscope (Leica TCS-SP2 AOBS).

Construction of Ran Eukaryotic Expression Vector and Transfection

To clone the full-length Ran coding sequence, primers containing specific restriction enzyme sites were designed. Total RNA was extracted from cultured BMECs, and cDNA synthesis of Ran mRNA was performed. The PCR product was inserted into pMD18-T plasmids (TaKaRa) and identified using EcoRI and SalI restriction enzymes, followed by DNA sequencing.

The Ran gene was cloned into the pGCMV-IRES-EGFP vector (GenePharma Co. Ltd, China) Using the upstream primer 5'-**CGGAATTCGAAGGAACGCCGCGATGG-**

3' (EcoRI) and the downstream primer 5'-**GCGTCGACCAGGTCATCATCCTCATCCGGG**-3' (Sal I), Transient transfection was performed according to the previous study by Lu *et al*., 2013. In brief, LipofectamineTM 2000 (LF2000, Invitrogen) was used to transfect BMECs with pGCMV-IRES-EGFP (empty plasmid) and pGCMV-IRES-EGFP-Ran (recombinant plasmid) in accordance with the manufacturer's instructions. Nontransfected cells were conducted as a blank control in the same way as transfection. Prior to cell treatment, BMECs were cultured for 24 hours at 37°C with serum-free media and antibiotics.

Stable transfection of pGCMV-IRES-EGFP-Ran in BMECs

Stable transfection of the Ran gene was conducted as described by Liu et al. (2012). BMECs transfected with pGCMV-IRES-EGFP-Ran were cultured in DMEM:F12 medium supplemented with 10% FBS and 600 μg/mL Geneticin (G418) (Gibco) for three weeks to obtain stably transfected clones.

Western Blot Analysis

The expression of Ran, STAT5, p-STAT5, mTOR, p-mTOR, and β-actin was analysed by Western blot using the conventional methods outlined by Huang *et al*. 2012 and Huang *et al*. 2013, To put it briefly, a 10% SDS-PAGE gel was used to run complete cell lysate (lysis buffer and including protease inhibitors)_, which included around 30 μg of protein, before it was transferred to a nitrocellulose membrane (Bio-RAD, Shanghai, China). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, then incubated with primary antibodies specific for mTOR, p-mTOR, STAT5, p-STAT5, Ran, and β-actin. After washing, membranes were incubated with HRP-conjugated secondary antibodies (1:1000, ZSGB-BIO, Beijing China); the membrane was probed with primary antibodies specific for the following antibodies: mTOR, p-mTOR (Beverly Cell Signalling Technology, MA, USA), β-actin (Santa Cruz, CA, USA), STAT5, p-STAT5, and Ran. Using Super ECL plus (Apply GEN, Beijing, China), HRP-labeled secondary antibodies were detected chemiluminescently. Western blot greyscale scans were examined using Hayward, USA's Glyko Band Scan 5.0 program.

Cell Viability Assay

With a few minor adjustments, the CASY-TT analysis equipment (Schärfe equipment GmbH, Germany) was used to measure cell viability in accordance with the manufacturer's instructions. 17 Cursor locations were adjusted to 11.75 to 50.00 μm (evaluation cursor) and 7.63 to 50.00 μm (normalisation cursor) after calibration with both dead and active BMECs. Before testing, BMECs were digested with trypsin and then diluted (1:100) with CASY electrolyte solution. Three 100 μL aliquots were analyzed in a sample.

β-casein secretion

Tong *et al.* 2012 reported that the secretion of β-casein in the cell-free supernatant of the culture was detected by HPLC.

Bioinformatics Analysis

The NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) provided the mRNA sequence of *Bos taurus* RAN, and the STRING web tool (https://string-db.org/) was used to predict protein-protein interactions and analyse the structure of the RAN protein.

Statistical Analysis

Using SPSS 13.0 (Chicago, USA), all data were presented as mean ± (SD) (n = 3) and statistical significance was checked in ANOVA ($P < 0.05$ and $P < 0.01$, respectively).

RESULTS AND DISCUSSION

All cultivated BMECs tested positive for CK18 and β-casein expression using confocal laser scanning microscopy (Figure 1A). This demonstrated that these main BMECs were pure, capable of breastfeeding, and suitable for use in further research.

RT-PCR was used to amplify the Ran gene, and the resultant sequence exactly matched the Ran mRNA sequence found in GenBank. EcoRI and SalI digestion and sequencing confirmed successful cloning of the 776 bp fragment into the pGCMV-IRES-EGFP vector. According to Western blot detection, after transient Ran activity (Figure 1B, C) and stable transfection of BMECs (Figure 1D, E), we found a considerable increase in the expression of mTOR, p-mTOR, Stat5, and p-Stat5. These findings imply that Ran could positively regulate the mTOR and Stat5 signalling pathways. Following both temporary and permanent Ran overexpression,

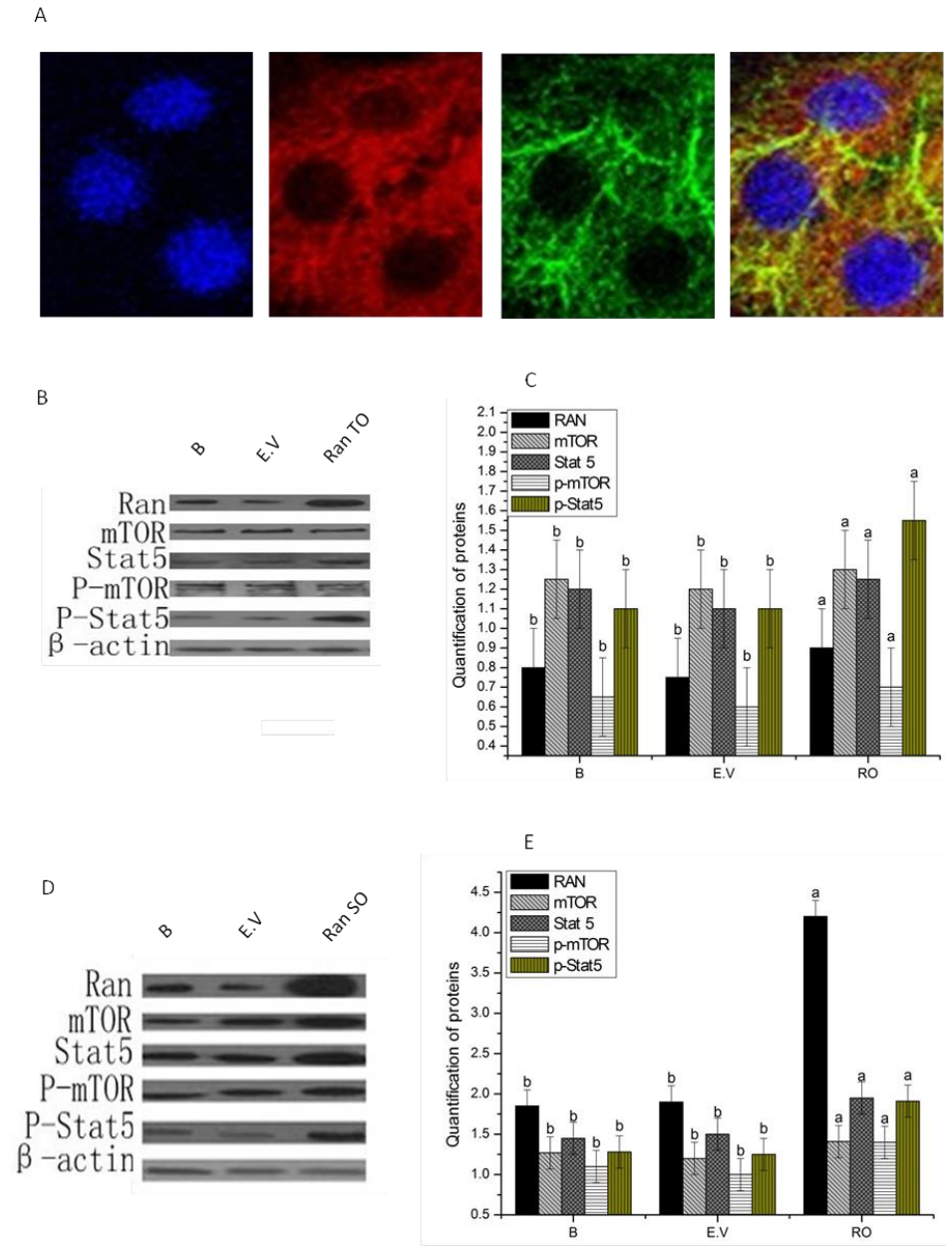

Figure 1. shows the Western blot detection of (mTOR, p-mTOR, Stat5, and p-Stat5) expression in BMECs after Ran transient and stable overexpression.

(A) CK18 and β-casein expression in BMECs was detected by immunofluorescence (400×). Red indicates β-casein stained with primary antibody and TRITC-labeled secondary antibody, green indicates CK18 stained with primary antibody and FITC-labeled secondary antibody, and blue indicates nuclear DNA stained with DAPI. (B) Western blot analysis revealed the expression of the identified proteins after a brief overexpression of Ran; β-actin served as a loading control. Three kinds of cells were created: EV (empty vector group); B (blank); and Ran TO, Ran transient overexpression. (C) Using greyscale scanning of (B), the relative protein of interest/β-actin ratio is quantified. (D) Western blot analysis revealed the expression of the specified proteins after a brief overexpression of Ran; β-actin served as a loading control. Three groups of cells were created: EV (empty vector group); B , normal cells (blank); and SO (Stable Overexpression). (E) Greyscale scanning of (D) is used to quantify the relative folded protein of interest/β-actin ratio. Mean ± SE (n = 3) is shown by the values. *P < 0.05 and **P < 0.01 in comparison to the negative control group (EV). Different lowercase letters indicate significant differences.

β-casein secretion progressively increased because BMECs were cultivated in serum-free media for testing. We saw increased β-casein secretion at several cultures time periods after temporary transfection of the Ran gene, although this was not statistically significant (Figure 2A). At various culture durations (12 and 24 hours), however, the β-casein secretion was noticeably greater than the control after stable transfection (Figure 2B). These results suggest that Ran can actively regulate milk protein production in BMECs.

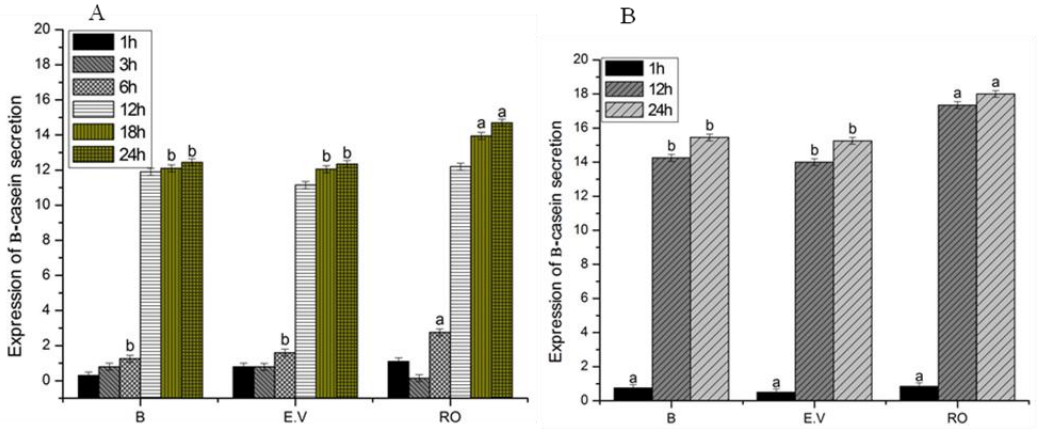

Figure 2. Shows the HPLC detection of β-casein secretion after both temporary and permanent Ran overexpression.

(A) Following temporary overexpression of Ran, the β-casein concentration of BMECs was measured by HPLC at various intervals. Three kinds of cells were created: EV (empty vector group); B (blank); and Ran TO, Ran transient overexpression (B) Following stable overexpression of Ran, the β-casein content of BMECs was measured by HPLC at various intervals. The cells were separated into three groups: Ran SO, or Ran stable overexpression; EV, or empty vector group; and B, or normal cells (empty). Mean ± SE (n = 3) is shown by the values. When compared to the negative control group (EV), Different lowercase letters indicate significant differences. *P < 0.05 and **P < 0.01.

We found that the cell survival at all culture durations was much greater than that of the control cells after both transient and stable transfection of the Ran gene (Figure 3 A and B). Cell viability reflects the capacity for growth and indicates the general health of BMECs. These results suggest that Ran overexpression enhances cell proliferation and does not negatively affect BMEC viability.

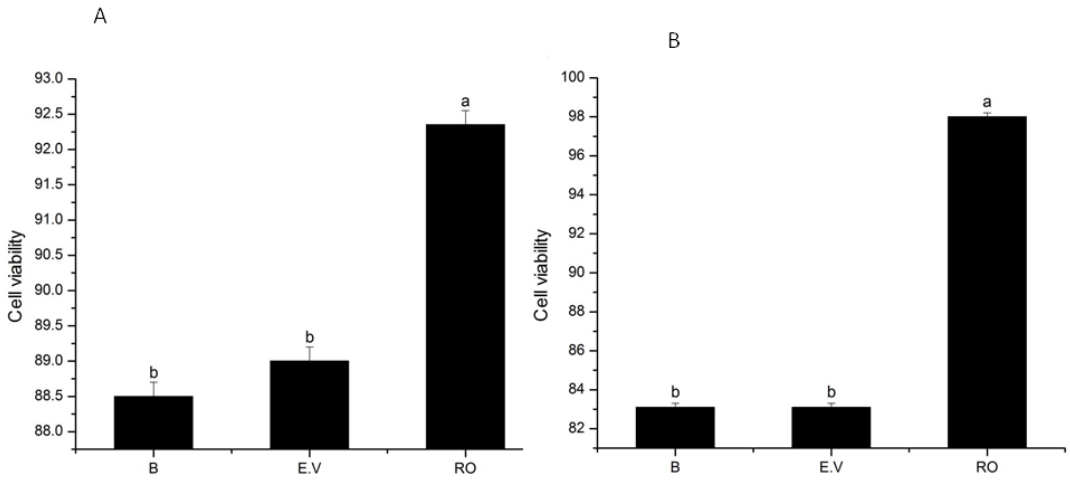

Figure 3. The CASY cell viability analyser was used to test the cell viability after both temporary and permanent overexpression of Ran.

(A) The CASY cell viability analyser was used to test the viability of BMEC cells at various intervals after the transient overexpression of Ran. Three kinds of cells were created: EV (empty vector group); B (blank); and Ran TO (Ran transient overexpression). (B) The CASY cell viability analyser was used to test the BMEC cell viability at various intervals after stable overexpression of Ran. Three kinds of cells were created: EV (empty vector group); B (normal cells; blank); and Ran SO (stable overexpression of Ran). Mean ± SE (n = 3) is shown by the values. *P < 0.05 and **P < 0.01 in comparison to the negative control group (EV). Different lowercase letters indicate significant differences.

Figure 4 illustrates the proposed mechanism by which Ran protein regulates milk protein synthesis in BMECs. Based on experimental findings and previous literature, Ran may directly activate the mTOR signaling pathway through interaction with nucleoporin 50 (NUP50), forming a regulatory gate that controls macromolecule transport between the nucleus and cytoplasm (Matsuura *et al*., 2005). Also, Smitherman *et al*, (2000) found (NUP50) interactions with Importin subunit beta-1 (KPNB1) which interactions with Cyclin-dependent kinase inhibitor 1B (CDKN1B) is regulated by an activating phosphorylation, inhibitory phosphorylation, associated with inhibitory proteins and interactions with AKT. On the other hand, JAK-STAT signaling integrates with the mTOR pathway, where phosphorylated STATs enter the nucleus via the Ran-dependent import mechanism. Once inside, dimeric STATs bind DNA regulatory sequences, activating transcription of target genes (Rawlings et al., 2004), which in turn leads to mTOR activation and regulation of protein synthesis.

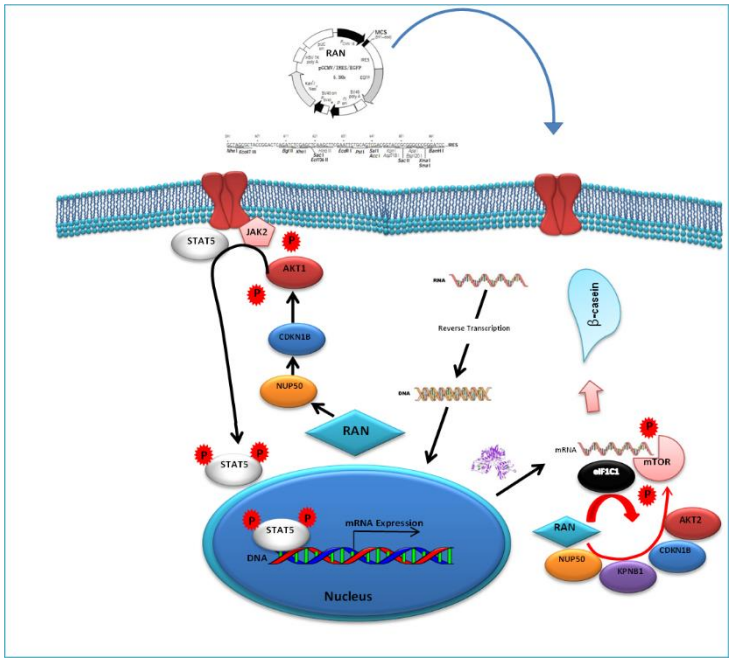

Figure 4. Diagram showing how the Jak2/Stat5 and mTOR signalling pathways are used by Protein Ran to control the production of milk proteins in bovine mammary epithelial cells.

In this experiment, we found that stable expression of Ran had more pronounced effects than transient transduction; this suggests that stable gene transduction can be used to study gene function. The present study found that Ras family members are involved in the regulation of several mTOR signaling pathways. In this study, the expression levels of mTOR and p-mTOR in Ran-overexpressing cells were significantly increased, suggesting that Ran can positively regulate the mTOR pathway in BMECS. The function of mTOR in controlling milk protein synthesis has been highlighted by recent research in ruminants and rodents (Li et al., 2025). For gene transcription, protein translation, and cell division, mTOR—a serine/threonine protein kinase—integrates various external signals, including growth factors, energy status, and nutrients (Yang et al., 2022; Ma and Blenis, 2009). These findings mean that Ran uses the mTOR signalling pathway to favorably manipulate the production of milk proteins. Ran plays a key role in nuclear membrane formation, nucleoplasm maintenance, DNA replication, and spindle assembly, and is also a critical regulator of cell proliferation. Recent studies have shown that Ran participates in multiple physiological processes, including transcriptional regulation and protein synthesis (Yudin and Fainzilber, 2009). Ran activity, that is managed by way of the asymmetric localization of Ran activating protein 1 (RanGAP1) inside the cytoplasm and Ran guanine nucleotide trade thing (Ran GEF/RCC1) within the nucleus, maintains nuclear and cytoplasmic compartmentalization in eukaryotic cells (Asakawa *et al.*, 2011). PI3K, Akt, mTOR kinases, and Ran proteins power H2O2-induced mitogenic indicators in kind II number one

pneumocytes (Radisavljevic et al., 2004). Ran controls ciliogenesis and ciliary protein delivery (Fan *et al*., 2011).

Important elements in milk production are the quantity and activity of BMECs (Boutinaud *et al*., 2004). Consequently, we looked into Ran's impact on cell division next. Using CASY studies, we discovered that cells overexpressing Ran had considerably higher Stat5 expression. Furthermore, there was a considerable increase in its phosphorylation state, suggesting that Ran has the ability to favourably control the Stat5 signalling pathway. Mammary gland differentiation, proliferation, and CSN2 production are all significantly impacted by Stat5 (Cimica *et al*., 2011). Among them, Stat5 is crucial to the BMECs' JAK2/STAT5 signalling pathway (Buser *et al.,* 2007). Ran must hydrolyse GTP in order for Stat1 to be unclearly imported. Stat1's active nuclear import depends on tyrosine phosphorylation, whereas its controlled nuclear import depends on Ran4/GT4 and its GTPase activity (Sekimoto *et al*., 1996). Ran transports STAT3 into the nucleus (Spozarski & Resendes, 2022). Activated Stat5 enters the nucleus and activates the transcription of many milk protein genes by binding to conserved DNA sequence motifs in their promoters (Zhu *et al*., 2021). These findings imply that Ran uses the Stat5 signalling pathway to actively control the production of milk proteins.

CONCLUSION

To the best of our knowledge, this is the first evidence in BMECs; Ran favorably regulates milk protein production via influencing the mTOR and Stat5 signalling pathways.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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